

A *Corynebacterium pseudotuberculosis* bacterin with muramyl dipeptide induces antibody titers, increases the time of onset, and decreases naturally occurring external abscesses in sheep and goats

K.A. Brogden^{a,*}, J.S. Glenn^b, N. East^b, F. Audibert^c

^aNational Animal Disease Center, ARS, USDA, Ames, IA 50010, USA

^bSchool of Veterinary Medicine, University of California, Davis, CA 95616, USA

^cVACSYN S. A., "Les Chevrons" 33, boulevard du General Martial Valin, 75015 Paris, France

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Abstract

Lambs from a university sheep flock and kids from a commercial goat dairy were injected with a bacterin containing *Corynebacterium pseudotuberculosis* (1 mg whole cells and 50 µg muramyl dipeptide in 10% light mineral oil) twice i.m. in the thigh, 1 month apart. All animals were then exposed to naturally infected adults under field conditions. Serum antibody titers to *C. pseudotuberculosis*, determined regularly up to 19 months in all animals vaccinated in 1990 and up to 7 months in all animals vaccinated in 1991, rose sharply after vaccination and remained higher ($P < 0.05$) in vaccinated animals after that. Lambs and kids born in 1990 were watched for 28 months and 21 months, respectively, for development of naturally occurring external abscesses and lambs and kids born in 1991 were watched for 15 months and 8 months, respectively, until the project was ended. Vaccine efficacy was assessed by both the period of time for vaccinated animals to develop abscesses (i.e. time-to-infection) and the final number of vaccinated animals with abscesses. Abscesses occurred in 9/22 non-vaccinated lambs (time-to-infection 478 ± 78 days) and in 4/21 (NS at $P < 0.05$) vaccinated lambs (time-to-infection 665 ± 42 days, NS at $P < 0.05$). Lack of significance was due primarily to the low numbers of lambs with abscesses remaining in the trial after attrition losses. Abscesses occurred in 14/82 non-vaccinated kids (time-to-infection 483 ± 35 days) and in 7/75 (NS at $P < 0.05$) vaccinated kids (time-to-infection 595 ± 20 days, $P < 0.05$). Local injection site reactions (e.g. inflammation, abscess formation) or systemic reactions (e.g. lethargy) due to bacterin administration were not seen in any animal.

Keywords: Caseous lymphadenitis; Vaccine; Muramyl dipeptide; Sheep; Goat

1. Introduction

Abscesses are commonly found externally on sheep and goats and occasionally in the visceral cavities of sheep (Ayers, 1977; Ashfaq and Campbell, 1979). These abscesses often result after a subcutaneous infec-

tion by a variety of bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Actinomyces pyogenes*, *Rhodococcus equi*, *Moraxella* species, and *Corynebacterium pseudotuberculosis* (Ashfaq and Campbell, 1979). Organisms then migrate in interstitial fluid from the site of infection to peripheral lymph nodes, thoracic lymph nodes, and occasionally the lung. Subcutaneous abscesses in goats can also occur (Ayers, 1977) and abscesses in sheep

* Corresponding author. Tel: +1-515-2398593. Fax: +1-515-2398458.

can also result from the mechanical transmission of organisms in caseous exudate from one sheep to another during shearing. *Corynebacterium pseudotuberculosis* is usually the most common organism isolated from abscess exudate and is responsible for the specific disease known as caseous lymphadenitis (CLA).

In abscess exudate and necrotic debris, *C. pseudotuberculosis* remains protected behind walls of fibroblasts and collagenous tissue (Ellis, 1988). This environment makes the organism refractory to treatment with antibiotics or immunotherapy. Therefore, vaccination of young lambs and kids before infection may be the only effective means of prophylaxis. Although *C. pseudotuberculosis* is a facultative intracellular pathogen, vaccines containing inactivated organisms (Cameron et al., 1972; Brogden et al., 1984) or culture supernatants (Nairn et al., 1982; Brown et al., 1986; Ellis et al., 1991b) have induced partial protection. In a previous study (Brogden et al., 1990), a water-in-oil emulsion bacterin containing inactivated whole cells of *C. pseudotuberculosis* and muramyl dipeptide was developed and protected lambs in laboratory trials. Here, we examined the efficacy of this bacterin for preventing naturally occurring external abscesses in lambs from a university sheep flock and kids from a commercial goat dairy.

2. Materials and methods

2.1. Animals

Two sites were chosen for the field evaluation of bacterin efficacy. The first site was at the California Polytechnic State University, San Luis Obispo, California. The California Polytechnic State University maintains a flock of purebred Suffolk and Hampshire sheep that are used in the university teaching and research programs. This flock has a long history of problems with *C. pseudotuberculosis*. Before the trial, serologic analysis showed that 77/117 ewes (66%) had antibody titers ($> 1:80$) to *C. pseudotuberculosis*. Lambs are born in January (Hampshire) and February (Suffolk). From these, 134 6-week-old lambs were selected in 1990 and 68 6-week-old lambs were selected in 1991. The second site was a commercial goat dairy in the San Joaquin valley of California. This

herd (of approximately 400 does) also has a long history of problems with *C. pseudotuberculosis*. By the end of the first lactation most does have subcutaneous nodules and enlarged peripheral lymph nodes, typical of *C. pseudotuberculosis* abscesses. Kids are born during three periods: October–November, January, and March. From these, 264 6-week-old kids were selected in 1990 and 133 6-week-old kids were selected in 1991.

2.2. Bacterin preparation

Corynebacterium pseudotuberculosis ATCC 19410 was grown on Tween–Albumin agar as previously described (Brogden et al., 1990). At 24 h, organisms were removed from the agar surface with distilled water, collected by centrifugation for 30 min at $4080 \times g$ at 4°C , and washed twice in distilled water. Organisms were washed once in 50% acetone, once in 100% acetone, twice in ethyl ether, and air dried.

Stock suspensions of bacterial cells (1.17 mg ml^{-1}) and synthetic muramyl dipeptide (MDP; 2-acetamido-2-deoxy-3-*O*-(*D*-2-propionyl-*L*-alanyl-*D*-isoglutamine)-*D*-glucopyranose; 1.25 mg ml^{-1}) were prepared in 0.14 M NaCl containing 0.2% sorbitan mono-oleate and 0.1% formalin. Bacterial cell suspension (0.86 ml per dose) and MDP (0.04 ml per dose) were added to light mineral oil (0.10 ml per dose) and emulsified as previously described (Brogden et al., 1990). Final concentrations (per ml dose) were 1.0 mg bacterial cells and $50 \mu\text{g}$ MDP in 10% oil. Bacterins were prepared at the National Animal Disease Center 3 days before the scheduled vaccination, checked for sterility by standard culture technique, and shipped to the School of Veterinary Medicine, University of California, Davis, California.

2.3. Immunization of animals

Before vaccination, all animals were bled and examined for external abscesses. Each year of the trial, lambs and kids were divided by sex. Using a table of random numbers, half the males and half the females were injected twice i.m. with 1 ml of the bacterin in the thigh, 1 month apart. The remaining non-vaccinated lambs and kids served as flock and herd contact controls.

2.4. Serology

All lambs and kids were bled monthly for 4 months after the initial vaccination and then quarterly after that. Agglutination antibody titers were determined at the National Animal Disease Center with a microagglutination assay (Menzies and Muckle, 1989). This assay uses *C. pseudotuberculosis* grown in broth containing 0.003% triphenoltetrazolium chloride as the antigen. The microagglutination test is not a good indicator for predicting disease in infected sheep and goats as antibody titers do not often correlate with lesion scores. However, the test is rapid, easy to use, suitable for determinations on large sample sizes, can discern the vaccination response, and can discriminate between vaccinated and non-vaccinated animals. Negative animals generally have titers ranging from 1:5 to 1:80. Higher titers ($> 1:80$) show exposure of the animal to the organism or vaccine. Lambs vaccinated in 1990 were bled for 19 months, lambs vaccinated in 1991 were bled for 6 months, kids vaccinated in 1990 were bled for 18 months, and kids vaccinated in 1991 were bled for 7 months.

2.5. Determination of bacterin efficacy

All lambs were exposed by natural contact with infected adult sheep already in the flock. Kids, isolated from adults until the start of the breeding season, were added to the milking herd after freshening. Sheep and goats in both groups were examined at each bleeding for external abscesses. The date any animal developed a subcutaneous nodule, enlarged peripheral lymph node, or other naturally occurring external abscess was recorded. When possible, abscesses were lanced and abscess material was sent to the University of California Veterinary Diagnostic Laboratory System for microbiological finding and diagnoses. Lambs vaccinated in 1990 were watched for 28 months, lambs vaccinated in 1991 were watched for 15 months, kids vaccinated in 1990 were watched for 21 months, and kids vaccinated in 1991 were watched for 8 months.

2.6. Statistics

Agglutination titers were determined twice on each serum sample, converted to logarithm₂, and averaged. The serologic response of animals after vaccination and

the difference between vaccinated and non-vaccinated lambs and kids were determined with the SAS Institute Inc. (1985). All serologic data, including titers of animals lost to attrition part way through the trial, were included. Therefore, trial numbers decline with time. The effect of vaccination on the onset and prevalence of naturally occurring external abscesses in animals after the trial was analyzed by ANOVA, chi-square, and Fisher's Exact test (SAS Institute Inc., 1985).

3. Results

3.1. Serology

Serum antibody titers to *C. pseudotuberculosis* were higher ($P < 0.05$) in vaccinated animals throughout the trial period. In lambs vaccinated in 1990 (Fig. 1), titers rose sharply after vaccination and continued gradually upward. At 19 months, titers in vaccinated lambs ranged from 1:1280 to 1:163 840. A similar trend was observed in lambs vaccinated in 1991 (Fig. 1).

In kids vaccinated in 1990 (Fig. 2), titers also rose sharply after vaccination and remained higher ($P < 0.05$) than non-vaccinated kids. At 18 months, titers in vaccinated kids ranged from 1:160 to 1:163 840. A similar trend was observed in kids vaccinated in 1991 (Fig. 2).

By 19 months all non-vaccinated lambs had titers greater than 1:160 (range 1:160–1:10 240) and by 18 months, 91% non-vaccinated kids had titers greater than 1:80 (range 1:80–1:81 920). This increase in titer probably resulted from exposure to *C. pseudotuberculosis* contracted from the adults in the flock or herd.

3.2. Bacterin efficacy

After the trial, 43 lambs remained in the 1990 group and 53 lambs remained in 1991 group. As part of the flock management program, ewe lambs born each year enter the flock as replacements, are sold as outside replacements, or are slaughtered at market weight. Ram lambs either enter the flock as replacements, are kept until 18 months of age and sold as breeding rams, or are also slaughtered at market weight. Similarly, 157 kids remained in the 1990 group and 89 kids remained in the 1991 group. As part of the herd management program, kids born each year enter the herd as replace-

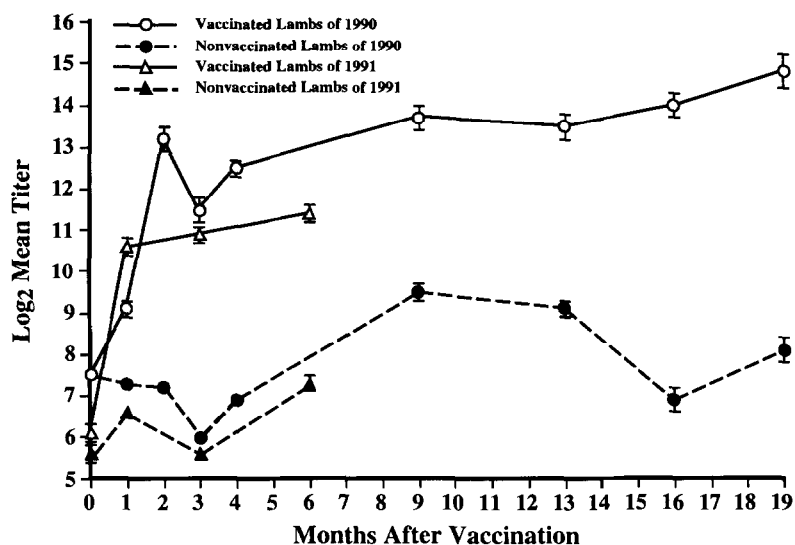


Fig. 1. Comparison of the agglutinating antibody titers of non-vaccinated lambs and lambs vaccinated with a *Corynebacterium pseudotuberculosis* bacterin containing MDP. Shown are the means \pm SE of Log₂ transformed antibody titers.

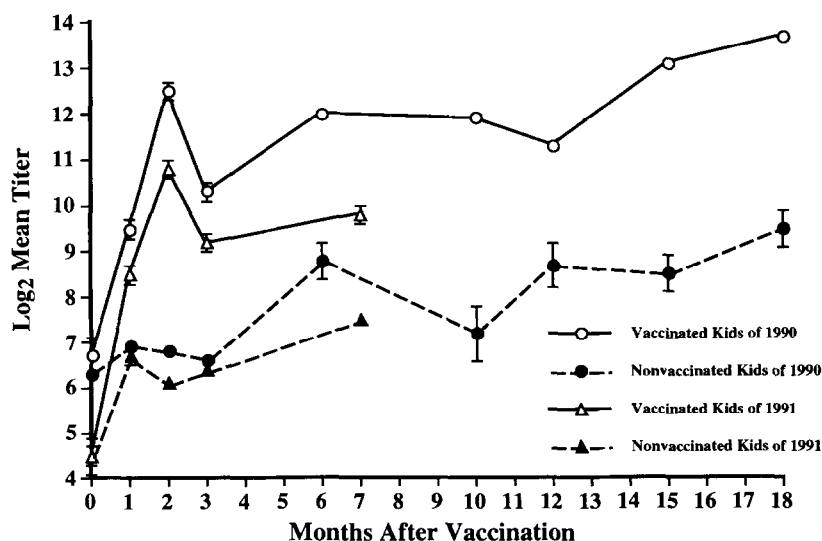


Fig. 2. Comparison of the agglutinating antibody titers of non-vaccinated kids and kids vaccinated with a *Corynebacterium pseudotuberculosis* bacterin containing MDP. Shown are the means \pm SE of Log₂ transformed antibody titers.

ments or are slaughtered at market weight for ethnic consumption. In both groups of animals, an extensive follow-up history was not possible.

At 28 months post 1990 vaccination, natural external abscesses occurred in 40.1% (9/22) non-vaccinated lambs (time-to-infection 478 ± 78 SE days, range 31–738 days; Table 1). Abscesses occurred in 19.0% (4/

21; NS at $P < 0.05$) vaccinated lambs (time-to-infection 665 ± 42 SE days range 591–738 days, NS at $P < 0.05$). Although the difference in time-to-infection between non-vaccinated and vaccinated lambs was about 6.1 months, there were not enough infected lambs in either group to show significant differences.

Table 1
Number of abscesses observed in vaccinated and control lambs and kids

Group		Vaccination status	No. animals with abscesses (probability) ^a	Days-to-infection (probability) ^b
Lambs	(1990)	Yes	4/21 (0.114)	665 ± 42 ^c (0.156)
		No	9/22	478 ± 78
	(1991)	Yes	0/28	0
		No	1/25	346
Goats	(1990)	Yes	7/75 (0.178)	595 ± 20 (0.044)
		No	14/82	483 ± 35
	(1991)	Yes	0/45	0
		No	0/44	0

^aFisher's exact test (SAS Institute Inc., 1985).

^bANOVA (SAS Institute Inc., 1985).

^cMean ± SE.

At 15 months post 1991 vaccination, abscesses occurred in 4.0% (1/25; NS at $P < 0.05$) of non-vaccinated lambs (time-to-infection 346 days, NS at $P < 0.05$). Abscesses were not seen in vaccinated lambs.

At 21 months post 1990 vaccination, natural external abscesses occurred in 17% (14/82) non-vaccinated kids (time-to-infection 483 ± 35 SE days, range 174–637 days; Table 1). Abscesses occurred in 9.3% (7/75; NS at $P < 0.05$) vaccinated kids (time-to-infection 595 ± 19 SE days, range 540–637 days, $P < 0.05$). At 8 months post 1991 vaccination, there were no abscesses in either non-vaccinated or vaccinated kids.

There were no local injection site reactions (e.g. inflammation, abscess formation) or systemic reactions (e.g. lethargy) due to bacterin administration.

4. Discussion

A bacterin, containing 1.0 mg of *C. pseudotuberculosis* whole cells and 50 µg MDP in 10% oil, induced high agglutinating antibody titers in both lambs and kids. Titers rose sharply after vaccination and remained higher ($P < 0.05$) in vaccinated animals after that. Antibody titers gradually rose in non-vaccinated animals suggesting exposure to *C. pseudotuberculosis* transmitted from naturally infected adults. Vaccinated sheep and goats also took longer to develop naturally occurring external abscesses and had lesser number of animals with abscesses than non-vaccinated controls.

The lack of statistical significance between groups resulted from the low numbers of animals left after unexpectedly high attrition. Although the original numbers assigned to the vaccinated and control groups at the start of the trial were more than adequate, the numbers that remained a year later were not. This has been a problem in other CLA field vaccination trials (Menzies et al., 1991) particularly when the incidence of disease is low.

Unlike conventional vaccination-challenge trials, vaccine efficacy in field trials can only be determined by comparing the time-to-infection and the development of naturally occurring external abscesses on non-vaccinated animals with that seen on vaccinated animals. This is an adequate assessment in goats as most abscesses will be external but of limited value in sheep. False negative sheep, with internal abscesses, will not be detected. Also, animals may develop abscesses that do not contain *C. pseudotuberculosis* and therefore be unaffected by vaccination. At least one vaccinated sheep had an abscess that did not contain *C. pseudotuberculosis*.

The immunogen required to protect sheep and goats from CLA caused by *C. pseudotuberculosis* is not known. Vaccines containing whole cells (Cameron et al., 1972; Cameron, 1982), chemically extracted whole cells (Brogden et al., 1984), cell wall fractions (Cameron et al., 1969; Cameron, 1982; Brogden et al., 1984) and culture supernatants (Ellis et al., 1991b) with the phospholipase D exotoxin (Nairn et al., 1982; Brown

et al., 1986) have been developed and tested. The general efficacy of toxoid vaccines is questionable and thought to be due to the protective effects of anti-exotoxin antibody (Brown et al., 1986). Work by Ellis et al. (1991a) suggests that antitoxin antibodies probably have little impact on the recovery from infection once the organism is intracellular and disseminated from the site of entry. Sheep naturally exposed to *C. pseudotuberculosis* have demonstrable levels of antibodies to the exotoxin despite their disease status. Culture supernatant vaccines, containing exotoxin, have several other bacterial cell antigens that may be the actual protective components of the vaccine (Ellis et al., 1991b). These components may be carbohydrate or lipid antigens as antibodies in infected sheep did not react with any specific protein in SDS-PAGE immunoblots of corynebacterial culture supernatants. (Ellis et al., 1991a). Further evidence against exotoxin as a protective antigen was shown by Hodgson et al. (1992). Attenuated mutants of *C. pseudotuberculosis*, without the phospholipase D gene, elicited a strong humoral and cell-mediated immune response and protected sheep from wild-type challenge.

The protective antigen may be cell-associated (reviewed by Ayers, 1977). Whole cells of *C. pseudotuberculosis* induce partial protective immunity in sheep (Cameron et al., 1972; Cameron, 1982; Brogden et al., 1984). An immunogen, found in cell wall fractions, also induces protective immunity (Cameron et al., 1969; Cameron, 1982; Brogden et al., 1984). The degree of protection can be influenced by the dose of whole cells or cell walls and by the type of adjuvant (Cameron et al., 1972; Brogden et al., 1990).

CLA differs between sheep and goats in its clinical severity and distribution of lesions (Ayers 1977; Ashfaq and Campbell, 1979). The two species also differ in their response to vaccination against CLA (Menzies et al., 1991; Johnson et al., 1993). Generally, vaccines for intended use in sheep are ineffective when used in young goats (Johnson et al., 1993). However, in a previous field study (Menzies et al., 1991), 5 mg *C. pseudotuberculosis* whole cells (without MDP) protected sheep against CLA. The vaccine also induced some protection in goats. The vaccine containing 1 mg *C. pseudotuberculosis* whole cells and MDP in this present study increased the onset ($P < 0.05$) and number of naturally occurring abscesses in vaccinated kids. Also, no post vaccination swellings were seen in goats

that have been a problem in previous vaccination studies (Brown et al., 1986; Menzies et al., 1991; Johnson et al., 1993).

Corynebacterium pseudotuberculosis anti-exotoxin antibody may protect against exotoxin-mediated tissue damage and dissemination of the organism and opsonizing antibody may enhance phagocytosis by neutrophils and macrophages (Ellis 1988). However, cell-mediated responses (lymphoid and mononuclear phagocyte) may be essential in eliminating the organism (Ellis, 1988). Sheep infected with *C. pseudotuberculosis* develop pyogranulomas with mononuclear phagocytic infiltrates (Ellis, 1988). At the periphery of the lesion, large pulmonary alveolar macrophages are present in abscess walls and adjacent parenchyma. T lymphocytes, mainly CD4 + lymphocytes, and some macrophages are found close to the necrotic center. Gamma/delta lymphocytes and B lymphocytes are found in an outer part of the lymphocyte zone (Pepin et al., 1994).

Effective vaccination of sheep and goats against CLA may require activation of T helper cells and macrophages to kill intracellular organisms early after infection. Macrophages from *C. pseudotuberculosis* immune animals have several unique differences (reviewed by Ayers, 1977). These macrophages contain more lysosomes with substantially larger quantities of hydrolytic enzymes, show more efficient lysosome-phagosome fusion, and are bactericidal.

MDP can activate lymphoid and mononuclear cells. MDP is the smallest possible structure from mycobacteria (Ellouz et al., 1974) that retains the activity of Freund's complete adjuvant (i.e. activate T_H1 cell types; Audibert and Lise, 1983) without undesirable effects (i.e. lymphoid hyperplasia, delayed type hypersensitivity, and granulomatous reactions; Chedid, 1981). MDP can replace killed mycobacteria in Freund's complete adjuvant and can induce both humoral and cellular immunity (Warren et al., 1986) by acting on macrophages and B and T cells (Audibert and Lise 1993). MDP enhances carrier-specific, helper, CD4 T-cell function, particularly T_H1 cells, is mitogenic for B cells, and stimulates monocytes and macrophages through the production of IL-1. MDP-stimulated macrophages undergo a variety of metabolic, biochemical, and functional changes (Bahr and Chedid, 1986). These include increased adherence and spreading, increased chemotaxis, increased phag-

ocytosis, increased production of collagenase, PGE, cAMP, peroxides, and monokines, and activation of killing capacity.

Killed vaccines are the least efficacious in preventing infection by facultative intracellular bacteria, such as *C. pseudotuberculosis* (Collins and Campbell, 1982) and generally induce only a humoral response. By adding MDP, the immune response can be activated against intracellular bacterial infection (Woodard et al., 1980, 1981). Activation of *C. pseudotuberculosis*-specific humoral and cell mediated mechanisms with MDP would result in more efficient opsonization, phagocytosis, and intracellular killing.

5. Conclusion

In conclusion, we examined the efficacy of a bacterin for the prevention of naturally occurring abscesses in lambs and kids. This vaccine (Brogden et al., 1990) and a previous generation (Brogden et al., 1984), have induced antibody responses and protected lambs in experimental laboratory trials. However, field evaluations, such as this and a previous trial (Menzies et al., 1991), were not so clear-cut and only showed promising trends. This moderate success is not due to vaccine design but due to problems associated with the nature of the disease and field trial experimental design. First, CLA is a long-term chronic disease. The mean onset of abscesses in non-vaccinated animals is 15–16 months (Table 1). Second, not all naturally occurring abscesses are due to *C. pseudotuberculosis* infection. Those abscesses, probably caused by injury and those that do not contain *C. pseudotuberculosis* would not be eliminated by this vaccine. Third, there is difficulty in keeping animals long enough (Menzies et al., 1991). Due to the commercial nature of the herds and flocks, owners want to participate in the trial but do not want to hold animals for extended periods of time or follow-up on animals that have been sold. Finally, the success of this vaccine may not come from 1 year of use but from extended use. This would allow younger non-infected animals that are vaccinated to eventually replace older infected animals thus eventually eliminating the disease from the flock or herd.

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